



Research paper

Modulation of microRNAs in two genetically disparate chicken lines showing different necrotic enteritis disease susceptibility



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ABSTRACT

MicroRNAs (miRNA) play a critical role in post-transcriptional regulation by influencing the 3'-UTR of target genes. Using two inbred White Leghorn chicken lines, line 6.3 and line 7.2 showing Marek's disease-resistant and -susceptible phenotypes, respectively, we used small RNA high-throughput sequencing (HTS) to investigate whether miRNAs are differently expressed in these two chicken lines after inducing necrotic enteritis (NE). The 12 miRNAs, selected from the most down-regulated or up-regulated miRNAs following NE induction, were confirmed by their expressions in real-time PCR. Among these miRNAs, miR-215, miR-217, miR-194, miR-200a, miR-200b, miR-216a, miR-216b, and miR-429 were highly expressed in intestine derived from line 7.2, whereas, miR-1782 and miR-499 were down-regulated. In spleen, miR-34b and miR-1684 were the most up-regulated miRNAs in line 6.3. Notably, five out of six target genes, CXCR5, BCL2, GJA1, TCF12, and TAB3 were differentially expressed between line 6.3 and line 7.2, and showed suppression in the MD-susceptible chicken line. Their expression levels were conversely correlated with those of miRNA obtained from both HTS and quantitative real-time PCR.

These results suggest that some miRNAs are differentially altered in response to NE and they modulate the expression of their target genes in the two inbred lines. Collectively, HTS analysis of intestinal miRNAs from NE-afflicted inbred chickens showing different disease phenotypes led to the identification of host immunity genes regulated by miRNA. Future studies of the function of these miRNAs and their target genes in the host will lead to enhanced understanding of molecular mechanisms controlling host-pathogen interaction in NE.

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1. Introduction

Necrotic enteritis (NE) is an acute clostridial disease that causes weight depression, loss of appetite, and

sudden death (Hermans and Morgan, 2007). NE is commonly caused by *Clostridium perfringens*, a Gram-positive, rod-shaped, spore-forming, oxygen-tolerant anaerobe (Lee et al., 2011a,b). Due to the risk of transmission through the food chain, *C. perfringens* poses food safety hazards to humans (Van Immerseel et al., 2004; Olkowski et al., 2008). Although in-feed antibiotic growth promoters (AGPs) have controlled NE during the past decades effectively, NE has reemerged as a significant problem in many countries

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where the governmental regulation of AGP usage has been restricted in animal production (Van Immerseel et al., 2004; Lee et al., 2011a,b).

MicroRNAs (miRNAs) are small, non-coding RNAs that are 18–23 nucleotides in size and have been suggested to play a critical role in the regulation of gene expression. Over 60% the human genome has been shown to be under miRNA regulation (Friedman et al., 2009). miRNA function in diverse biological process such as endothelial inflammation and senescence (Qin et al., 2012), neuron development (Johnston and Hobert, 2003), tissue homeostasis (O'Neill et al., 2011) as well as many aspects of the immune response such as differentiation, selection, and activation of immune cells, cytokine responses, and intracellular signaling pathways (Maurel and Chevet, 2013; Sullivan et al., 2013; Bronevetsky and Ansel, 2013; Chen et al., 2013). Interestingly, the genes associated with immune responses are more highly targeted by miRNAs compared to the rest of the genome (Jeker and Bluestone, 2013). In chickens, microRNA expression has been studied in embryo developmental processes (Darnell et al., 2006; Hicks et al., 2008; Bannister et al., 2009), germ cell development (Lee et al., 2011c), immune organs (Hicks et al., 2009) and diseases (Burnside and Morgan, 2011; Tian et al., 2012; Stik et al., 2013). There is limited information concerning the mechanism(s) by which miRNAs regulate different immune target genes in NE infection in broiler chickens.

Understanding the molecular mechanisms that underlie miRNA-mediated post-transcriptional regulation of host immune response will facilitate the development of logical immunological control of NE. In this study, two genetically disparate chicken models, line 6.3 and line 7.2 of White Leghorn chicken that show Marek's disease virus (MDV)-resistant and -susceptible phenotypes, respectively (Bacon et al., 2000), were used to investigate differential miRNA expression and regulation of target genes in NE disease model that we previously described (Park et al., 2008; Lee et al., 2011b). Until now, no study to evaluate the responses to NE on these two chicken lines has been reported. In the present study, therefore, the differences in responses to NE and the levels of immune related gene expression were compared between lines 6.3 and 7.2.

2. Materials and methods

2.1. Animals and NE disease model

Two highly inbred White Leghorn chicken lines, 6.3 and 7.2, which were selected for their resistant (6.3) and susceptible (7.2) disease phenotypes to avian leukosis virus (ALV) and MDV, were used in this study. These two inbred lines have been maintained since 1931 (Bacon, 2002). The oral gavages with *E. maxima* strain 41A (3.0×10^3 oocysts/bird) at 14 days of age, and subsequent challenge infection with a field isolate of *C. perfringens* strain Del-1 (1.0×10^9 CFU/bird) at 18 days of age were carried out to induce NE as previously described by Lee et al. (2011b). Birds were fed a non-medicated commercial basal ration of 17% crude protein at 14 days of age. A standard grower diet containing 24% crude protein was given to chickens between day 18 and 20. NE infection was confirmed by

serum toxin levels (α -toxin and NetB toxin) and gut lesions as previously described (Lee et al., 2011b). All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee of USDA-ARS.

2.2. Total RNA extraction from spleen and small intestinal mucosal layer

Total RNA was isolated from spleen and intestine of five chickens per control or NE induced on d 20 post-hatch using TRIzol (Invitrogen, Carlsbad, CA) as previously described (Lee et al., 2010). The spleen collected from the chicken was frozen in liquid nitrogen and carefully homogenized using a pestle-mortar system. Intestines were cut longitudinally and washed three times with ice-cold Hanks' balanced salt solution (HBSS), which was prepared by adding 100 U/mL of penicillin and 100 mg/mL of streptomycin (Sigma, St. Louis, MO). The mucosal or inner layer was carefully removed using a cell scraper (Nunc, Thermo Fisher Scientific Inc., Roskilde, Denmark). Total RNA was extracted using TRIzol and processed to high-throughput deep sequencing.

2.3. High-throughput small RNA sequencing

Pooled small RNAs come from five chickens in the desired size range (18–30 nt) were eluted from denaturing 15% polyacrylamide gel and ligated with the 3' and 5' adaptors to generate a small RNA cDNA library, as previously described (Hafner et al., 2012). The cDNA was synthesized using 5' adaptor forward and 3' adaptor reverse primer, and the PCR products of small RNA fragment were sequenced by the Theragen Bio Institute (Suwon, Korea) using the HiSeq 2000 high throughput sequencer (Illumina, Inc., San Diego, CA).

2.4. Analysis of high-throughput sequenced data

The 50-nt sequence tags from HiSeq sequencing were subjected to a data cleaning process that included the removal of low quality sequence tags which do not meet a minimum quality criteria based on the sequence length and the mean quality, 5' primer and 3' primer contaminants, sequences without the insert tag, poly A tails, and tags that were shorter than 18 nt. Subsequently, a standard bioinformatics analysis was carried out to align or annotate the clean tags into the chicken genome by Rfam 11.0 (<http://rfam.sanger.ac.uk>) and the Genbank database. The differential expression pattern analyses of known miRNAs, or the associated mRNA gene targets were performed using miRBase 19.0 (<http://www.mirbase.org>), Mireap (<http://sourceforge.net/projects/mireap>), and TargetScanHuman (<http://www.targetscan.org>).

2.5. Expression analysis of known miRNAs for two chicken lines

The significance level (*P*-value) calculated using the *t*-test was used to determine NE-induced changes in the expression of miRNA in the spleen and intestine of both resistant and susceptible chicken lines. Normalization of

Table 1

miRNA primers for quantitative real-time PCR.

miRNA	miRNA sequence (5'-3')	* Forward primer sequence (5'-3')
gga-miR-215	AUGACCUAUGAAUUGACAGAC	GATGACCTATGAATTGACAGAC
gga-miR-217	UACUGCAUCAGGAACUGAUUGAU	TACTGCATCAGGAACGTGATGGAT
gga-miR-194	UGUAACAGCAACCUAUGUGGA	TGTAACAGCAACTCCATGTGGA
gga-miR-200a	UAACACUUGUCUGGUACGAUGU	TAACACTGCTGTAACGATGT
gga-miR-200b	UAAUACUCCUGGUAAUGAUGAU	GTAAATCTGCTGGTAATGATGAT
gga-miR-216a	UAAUCUCAGCUGGCAACUGUG	TAATCTCAGCTGCAACTGTG
gga-miR-216b	AAAUCUCUGCAGGCAAUGUGA	AAATCTCTGAGGCAAATGTGA
gga-miR-429	UAUAACUGUCUGGUAAUGCCGU	GTAAATACTGCTGTAATGCCGT
gga-miR-499	UUAAGACUUUGUAGUGAUGUUUAG	GGTTAACAGCTTGTAGTGATGTTAG
gga-miR-1782	ACAUUCAUUGGAGCAGGGACA	ACATTCATGGAGCAGGGACAA
gga-miR-34b	CAGGCAGGUAGGUUAGCUGAUUG	CAGGCAGTGTAGTTAGCTGATTG
gga-miR-1684	AAGUAUGAGGAAUUGGAGCUCU	AAGTATGAGGAAATGGAGCTCT

* Forward primer sequences were identical to the entire mature miRNA sequence. For miR-215, miR-200b, miR-429, and miR-499, nucleotide G was added to the 5' end of the remaining desired miRNA to adjust the Tm.

the expression levels of miRNA in both the spleen and intestine was performed the plotting Log2-ratio and Scatter Plot.

2.6. miRNA primer design

Quantitative real-time PCR (qRT-PCR) for miRNAs only requires design of the forward primer for the individual miRNA. The reverse primer is a universal primer provided with the NCode™ miRNA First-Strand Kit (Invitrogen). All known chicken miRNA sequences were obtained from miR-Base (<http://microrna.sanger.ac.uk/sequences/ftp.shtml>) and aligned using ClustalW (<http://www.genome.jp/tools/clustalw/>). Since all the analyzed miRNAs in this analysis are not highly homologous with the other miRNA sequences and are also GC-rich sequences, truncating the primer sequence was not necessary. Therefore, oligonucleotide primers for these miRNAs were designed by using full length of mature miRNA sequences. Primers were designed with a Tm range 50–55 °C. In some cases, one or more G was added to the 5' end of the remaining desired miRNA to adjust the Tm. However, it should be noted that the minimum length before adding G's should not be less than 15 nucleotides (Table 1).

2.7. miRNA expression analysis by quantitative RT-PCR

The NCode™ miRNA First-Strand cDNA Synthesis Kit and Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Invitrogen) were used to determine miRNA expression according to manufacturer's protocols. Briefly, 2.5 µg of pooled total RNA from five samples was combined with 5 µL of 5× miRNA reaction buffer, 2.5 µL of 25 mM MnCl₂, 1 µL ATP diluted in 1 mM Tris pH 8.0 from a stock of 10 mM ATP, 0.5 µL of poly A polymerase, and DEPC-treated water to give a final volume of 25 µL. The tube was incubated at 37 °C for 15 min to complete the poly(A) tailing procedure, which was immediately followed by first-strand cDNA synthesis. The mixture tube containing 4 µL of polyadenylated RNA, 1 µL of annealing buffer, and 3 µL of 25 µM Universal RT primer was incubated at 65 °C for 5 min and placed on ice for 1 min. Following this step, 10 µL of 2× First-Strand reaction mix and 2 µL of SuperScript™ III RT/RNaseOUT™ enzyme mix was added to the tube and preheated to 50 °C

for 50 min. To stop the reaction, the tube was incubated at 85 °C for 5 min. Synthesized cDNA was used to perform qRT-PCR as template. For one 20 µL reaction mix, the following components were added: 10 µL of 2× Power SYBR Green Master Mix (Life Technologies), 1 µL of 10 µM forward primer, 1 µL of 10 µM universal primer, 5 µL of 100 ng/µL cDNA, and DEPC-treated water to 20 µL. The standard cycling program for ABI 7500 (Life Technologies, Grand Island, NY) is as follows: 50 °C for 2 min to activate UDG, 95 °C for 2 min, and 45 cycles of 95 °C for 15 s, 60 °C for 30 s. Data from qRT-PCR was normalized relative to the expression of U1A (5'-CTGCATAATTGTGGTAGTGG-3').

2.8. Quantitative RT-PCR for mRNA expression analysis

To predict the miRNA target genes, the TargetScan-Human (<http://www.targetscan.org>) site was used. The potential target genes were further analyzed by qRT-PCR. In detail, 5 µg of pooled total RNA was treated with 1.0 unit of DNase I and 1.0 µL of 10× reaction buffer (Thermo Scientific) and then incubated for 30 min at 37 °C, followed by the addition of 1.0 µL of 50 mM EDTA and heating at 65 °C for 10 min. The DNase I treated RNA was used to synthesize cDNA using the Maxima First strand cDNA synthesis Kit (Thermo Scientific). Five micrograms of RNA was combined with 5× reaction mix, 2 µL of Maxima enzyme mix, and RNase-free water to a total volume of 20 µL. The mixture was first incubated at 25 °C for 10 min and then at 50 °C for 15 min, followed by heating at 85 °C for 5 min to terminate the reaction. To carry out qRT-PCR, 100 ng cDNA was added in a reaction mix including 10 µL of 2× Power SYBR Green Master Mix (Life Technologies), 0.5 µL of each primer and RNase-free water to a total volume 20 µL. The ABI 7500 system was used to perform a two-step qRT-PCR by following a standard cycling program: 50 °C for 2 min; 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 65 °C for 30 s. Each analysis was repeated three times. Standard curves were generated using log₁₀ diluted cDNA from pooled total RNA. Data from qRT-PCR was normalized relative to the expression of GAPDH. Oligonucleotide primers for target genes and the GAPDH control were designed based on sequences available in public databases (Table 2).

Table 2

Primers for quantitative real-time PCR.

Genes		Primer Sequence (5'-3')	Annealing Temp	Size (bp)	GenBank accession no.
GJA1	F	GTCCTCATGCTGGTAGTGTCTTGGTGTCT	65	119	NM_204586
	R	CTGTGGGAGTAGGGGTCGGTTITC			
CXCR5	F	AATCCACACCTACCGCAAAGCCAGAG	65	94	NM_001030912
	R	GAGATCAGGTAAGGTCAAGGACAG			
VEGFA	F	GGCTAGAATGTCTCCCTGTG	65	89	NM_001110355
	R	ATGTGCGCTATGCTGACTCTGA			
TAB3	F	CACCGCAAAGACCTGGGACTG	65	81	XM_416787
	R	GTGGGTGCTGGTTCTGAGATGGT			
TCF12	F	CTCGGGAAACCTGGAACAACCTACTA	65	120	NM_205375
	R	GGGGGCACCTTCTTACTTTCTTGTCT			
BCL2	F	GATGTGCGTCGAGAGCGTCAA	65	91	NM_205339
	R	GTGCAGGTGCCGGTTCAGGT			
GAPDH	F	TGCTGCCAGAACATCATCC	65	142	NM_204305
	R	ACGGCAGGTCAACAA			

2.9. Statistical analysis

Mean \pm standard error (S.E.) values for each group ($N=5$) were calculated, and differences between groups were analyzed by the Student's *t*-test using IBM SPSS software (SPSS 20.0 for Windows, Chicago, IL). Statistical differences were considered significant at $P < 0.05$.

3. Results

3.1. Analysis of known miRNAs expression by small RNA sequencing

Spleen and intestine miRNA expression is shown using the plotting tools Log2-ratio and Scatter Plot (Fig. 1A and B). Differential expression patterns could be classified into three types: up-regulation of miRNAs, down-regulation of miRNAs, and equal expression. In general, there was a distinct difference in the miRNA expression patterns observed in the two chicken lines, especially in intestine. High-throughput sequencing data of 149 known miRNAs obtained from intestine revealed that 137 miRNAs were differently expressed between two chicken lines, out of which 19 miRNAs are highly expressed in line 7.2, contrary to the pattern shown by 118 miRNAs (Table S1). Some of the most commonly observed miRNAs such as miR-215, miR-200b, miR-194, miR-200a, miR-216b, miR-216a, miR-217, miR-429, miR-499, and miR-1782 are indicated in Fig. 1A. In the spleen, a total of 139 known miRNAs were classified by comparing their expression levels between the two chicken lines (Table S2). In total, 28 miRNAs were observed to be either up-regulated or down-regulated and 111 miRNAs were equally expressed in both the chicken lines. Interestingly, miR-1684 and miR-34b were two most commonly down-regulated miRNA in the susceptible chicken line (Fig. 1B).

3.2. Quantitative RT-PCR to validate differentially expressed miRNAs

Twelve miRNAs from both spleen and intestine samples that showed the most significant change in expression were selected for further analysis by qRT-PCR (Table 3). The result indicated that miR-215, miR-217, miR-194,

miR-200a, miR-200b, miR-216a, miR-216b, and miR-429 were highly expressed in intestine derived from chicken line 7.2. In contrast, miR-1782 and miR-499 showed low expression levels in line 7.2 but highly expressed in line 6.3 (Fig. 2A and Table 3). The miR-34b and miR-1684 were equally expressed in the intestine (Table 3), but their expression was significantly altered in spleen where a higher expression level of these miRNAs was found in line 6.3 when compared with that in line 7.2 (Fig. 2B). Quantitative RT-PCR results for miRNA expression in the intestine and spleen of line 6.3 and line 7.2 chickens are summarized in Table 5 based on the Fig. 2A, B and Table 4.

3.3. Modulation of target gene expression by miRNAs

To understand the miRNA-regulation of target gene expression, we scanned the target genes for the 12 examined miRNAs (Table 4). Six putative target genes that have been previously shown to be involved in immune responses were analyzed by qRT-PCR for their expression (Fig. 3A and B and Table 6). In intestine, the expression of five out of the six target genes including CXCR5, BCL2, GJA1, TAB3 and TCF12 were suppressed in line 7.2. VEGFA was equally expressed in both line 6.3 and line 7.2 (Fig. 3A). The expression level of all these target genes was also examined in the spleen tissues from both chicken lines. Among the six predicted target genes, expression of CXCR5, BCL2, TCF12, and GJA1 was dramatically reduced in chicken line 7.2, whereas there was no significant change in the expression of VEGFA and TAB3 in both chicken lines (Fig. 3B). Indeed, expression of those mRNAs also has been validated in non-induced NE chickens that show a significant difference comparing to NE induced group.

4. Discussion

Necrotic enteritis (NE) is a major enteric disease of poultry caused by infection with *C. perfringens* (Oda et al., 2006; O'Brien et al., 2007; Olkowski et al., 2008). NE is widespread in broilers, imposing a significant economic burden on the poultry industry worldwide. Several publications have described the relationship between performance traits of commercial broiler breeds and their ability to mount immunological responses (Bacon et al., 2000; Collier et al.,

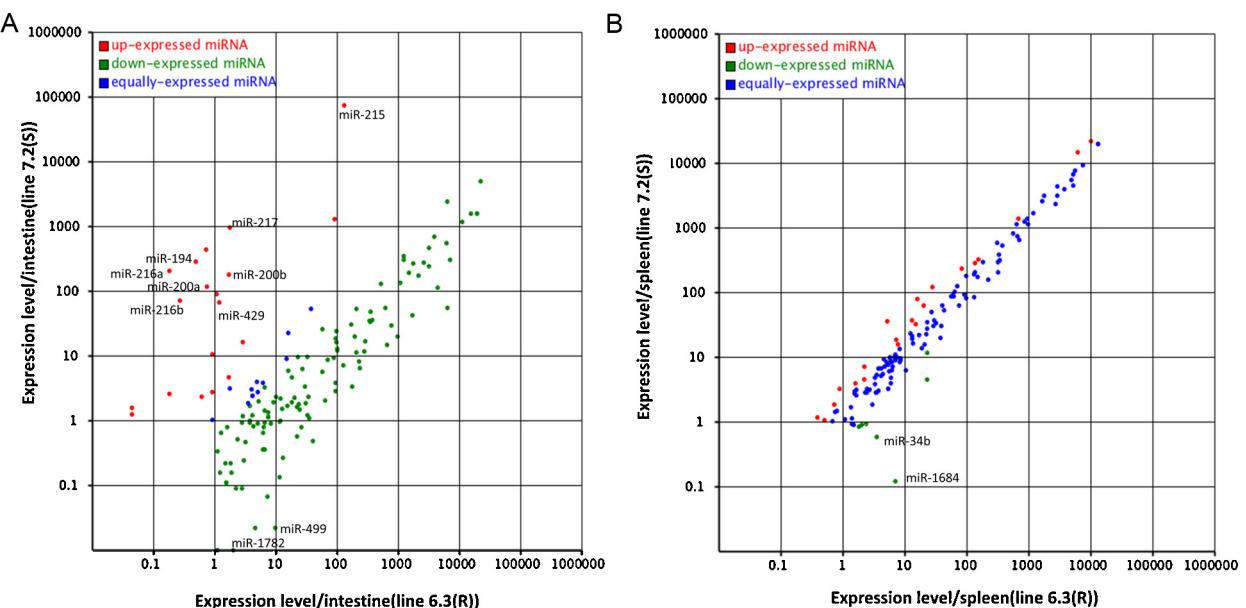


Fig. 1. Differential expression of known miRNAs in NE-afflicted chicken lines 6.3 and 7.2. Differential expression was observed in the intestine (A) and spleen (B). Red dots (fold change >1) indicate that miRNAs are highly expressed in line 7.2 in comparison with line 6.3, blue dots ($-1 < \text{fold change} < 1$) show miRNAs that are equally expressed in both lines, the green dots (fold change < -1) represent miRNAs that are down-regulated in line 7.2 in comparison with line 6.3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

Expression of up- and down-regulated microRNA (miRNAs) in spleen and intestine according to read count numbers from sequencing.

miRNA	Line 6.3_spleen	Line 7.2_spleen	Line 6.3_intestine	Line 7.2_intestine
gga-miR-215	1794	2170	5848	3354759
gga-miR-200b	125	76	77	8083
gga-miR-194	9	18	32	19856
gga-miR-217	99	39	79	42804
gga-miR-216b	6	10	12	3179
gga-miR-216a	17	3	8	9307
gga-miR-200a	75	35	33	5240
gga-miR-429	35	21	48	4000
gga-miR-1782	62	37	89	0
gga-miR-499	221	317	437	1
gga-miR-34b	147	24	40	2
gga-miR-1684	294	5	7	0

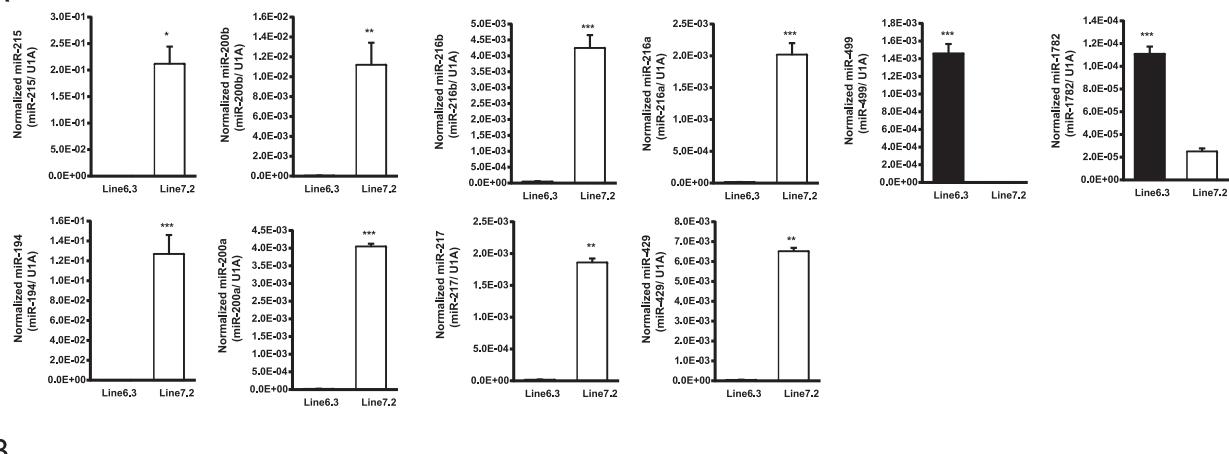
Table 4

Immune-related target genes for differentially expressed miRNAs.

miRNA	Symbol	Description
gga-miR-215	ALCAM	Activated leukocyte cell adhesion molecule
	CXCR5	Chemokine (C-X-C motif) receptor 5
gga-miR-217	TCF12	Transcription factor 12
	BCL11B*	B-cell CLL/lymphoma 11B (zinc finger protein)
gga-miR-194	TAB3	TGF-beta activated kinase 1/MAP3K7 binding protein 3
gga-miR-200a	TCF12	Transcription factor 12
gga-miR-200b	TNFRSF11B	Tumor necrosis factor receptor superfamily, Member 11b
	BCL2	B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein
	VEGFA	Vascular endothelial growth factor A
gga-miR-216a	TGFBR2	Transforming growth factor, beta receptor II (70/80 kDa)
gga-miR-216b	SOCS6	Suppressor of cytokine signaling 6
gga-miR-429	VEGFA	Vascular endothelial growth factor A
	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b
	BCL2	B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein
gga-miR-499	TCF12	Transcription factor 12
gga-miR-1782	GJA1	gap junction protein, alpha 1, 43 kDa
	SOCS6	Suppressor of cytokine signaling 6
gga-miR-34b	TCF12	Transcription factor 12
gga-miR-1684	MAPK6	Mitogen-activated protein kinase 6

* Only the predicted sequence is available.

A



B

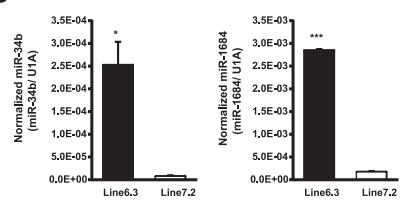
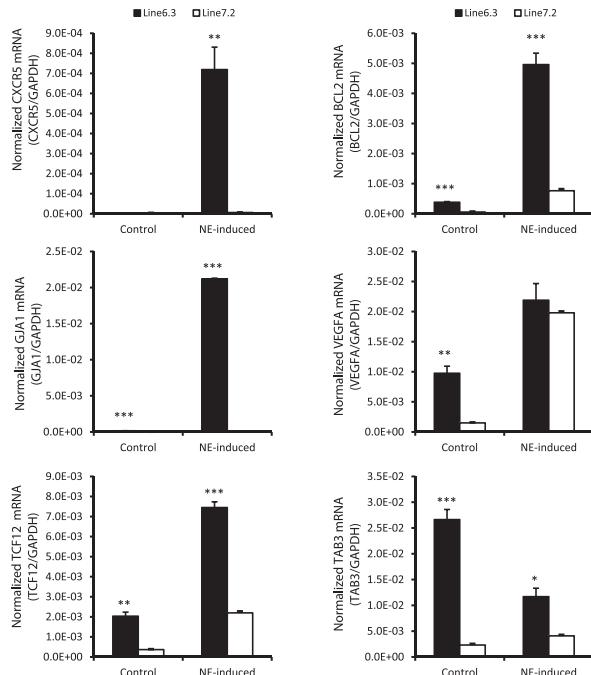


Fig. 2. Quantitative expression analysis of the most noticed miRNAs in the intestine (A) and spleen (B) derived from NE-afflicted lines 6.3 and line 7.2. The result from qRT-PCR was normalized to the expression levels of U1A. Significant differences in miRNA expression levels between line 6.3 and line 7.2 are indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Error bars indicate SE of technical replicate that was done in triplicate.

A (Intestine)



B (Spleen)

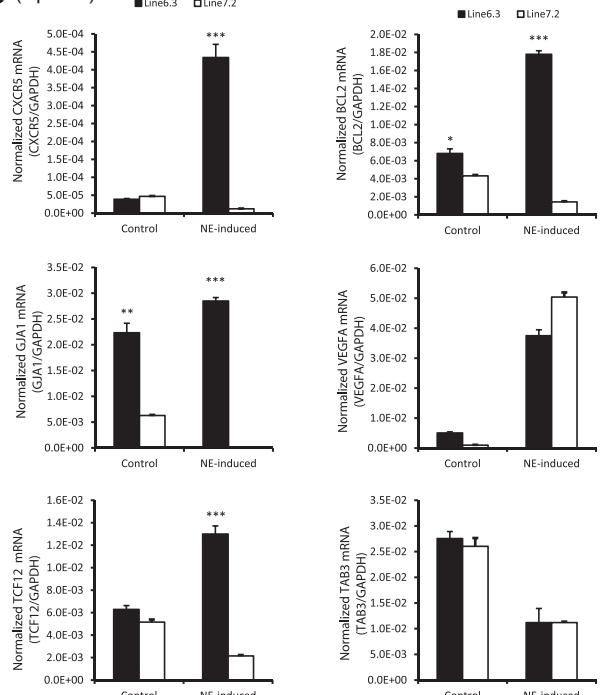


Fig. 3. miRNA target expression analysis in NE-afflicted and control chicken lines 6.3 and 7.2 in intestine tissue (A) and spleen (B) using qRT-PCR for miRNA target genes. mRNA expression levels were normalized to the expression levels of GAPDH. Significant differences between line 6.3 and line 7.2 are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars indicate SE of technical replicate that was done in triplicate.

2008). However, no information is available in the different chicken lines to experimental NE and underlying immune mechanisms associated with protection. Therefore, the current study was performed to identify genetic factors controlling host-pathogen interaction in NE.

A total of 89 million and 82 million small RNA sequence reads were obtained from intestinal mucosal layer and the spleen tissues of two NE-afflicted inbred chicken lines showing different responses to NE using next-generation sequencing (NGS). Some miRNAs, including miR-194, miR-217, miR-216b, miR-200a, miR-200b, and miR-429, were strongly up-regulated in the intestine derived from line 7.2. However, those miRNAs were not abundantly expressed in the spleen. In this regard, the results suggest that a tissue-specific change occurred in the expression of these miRNAs following NE induction. In the intestine, a large number of known miRNA expression were up-regulated in the line 6.3 (green dots). This result may suggest that the expression of many genes regulated by these miRNAs tend to be suppressed in line 6.3 in comparison with those of line 7.2. Once entering gastrointestinal tract, enteric pathogens including *C. perfringens* rapidly initiate intestinal epithelial response that involve alterations in the structure and function of the tight junction barrier as well as activation of the inflammatory cascade (Berkes et al., 2003). These interactions between bacteria and intestine might account for a huge number of differentially expressed miRNAs were found in intestine rather than in spleen. This finding also revealed that most of responses to NE largely occur in the intestinal mucosal layer.

BCL2 expression, which was suppressed in the intestine of line 7.2, has been suggested to function in apoptosis, since its down-regulation leads to apoptosis (Kinnally et al., 2006; Lynne Cassimeris et al., 2011; Zhu et al., 2013). miR-429, predicted to target *BCL2*, showed a high expression in intestine of line 7.2 (Fig. 2A and Table 5). In another report, overexpression of miR-429, significantly reduced apoptosis in the HT-29 cell line transfected with miR-429 mimics via targeting SOX2 gene (Li et al., 2013). Moreover, expression of *BCL2* in line 6.3 was increased following NE infection. We suggest that miR-429 and *BCL2* gene may biologically interact in NE-afflicted chickens, but the exact mechanism involved in the suppression of apoptosis in NE infection remains to be investigated.

Archambaud et al. (2012) has shown that miR-215 and miR-200b were repressed during *Listeria monocytogenes* infection. In contrast, our studies showed that miR-215 and miR-200b were up-regulated in the intestine of NE-afflicted line 7.2. This result suggests that the expression of miR-215 and miR-200b may vary depending on the pathogen type. Furthermore, miR-215 and miR-200b were predicted to target CXCR5 and *BCL2*, respectively. The expression level of these genes is inversely correlated with that of the corresponding miRNAs. Interestingly, CXCR5 has been known to direct the process of B-cell migration (Chan et al., 2013) as well as provide the essential signals for immunoglobulin (Ig) production to B cells working together with CD4+ T cells (Breitfeld et al., 2000). The role of *BCL2* as an apoptosis inhibitor has been extensively investigated, as mentioned earlier. Indeed, expression level of CXCR5 that is similar to *BCL2* shows a higher expression in

line 6.3 of NE-infected group in comparison with control group (Fig. 3A). These findings strongly support our conclusion that miR-215 and miR-200b may regulate CXCR5 and *BCL2*, respectively, during the immune response to NE.

VEGFA which plays an important role in angiogenesis (Przybylski, 2009) is regulated by miR-200b and miR-429. miR-200b has previously been shown to target VEGFA (Choi et al., 2011; Chan et al., 2013). However, expression of VEGFA in line 6.3 is obviously not different from line 7.2 (Fig. 3A). By comparison, miR-200b and miR-429 are highly expressed in line 6.3 compared with line 7.2 (Fig. 2A). Therefore, VEGFA was not regulated by miR-200b and miR-429. Surprisingly, VEGFA was stably expressed across two chicken groups. There was not statistically different between expression of VEGFA in NE-afflicted but control group. Consequently, we assumed that VEGFA may be regulated by another mechanism rather than by microRNA. This miRNA-independent mechanism may be involved in an increased sequestration of mRNA resulting in the formation of translationally inert complexes that are inaccessible to the translational machinery (Chang et al., 2012). Hence, there is neither an increase nor decrease in the mRNA expression level during gene regulation, as observed for VEGFA.

There was no correlation between predicted target gene regulation and miRNA expression in the case of *GJA1* and miR-1782 (Tables 5 and 6). Thus, the miRNAs regulating this gene was not identified. This could be explained by recent evidence that miRNAs may bind to 5'-UTR instead of 3'-UTR (Place et al., 2008; Tay et al., 2008), whereas TargetScan Human focuses on 3'-UTR, and hence resulting in missing of miRNAs that target *GJA1* gene. It is possible that a novel miRNAs may have contributed to *GJA1* regulation.

The up-regulation of miR-217 and miR-200a, and inversely, a down-regulation of miR-499 expression were examined in intestine of line 7.2 (Fig. 2A). These miRNAs were supposed to target the *TCF12* gene that plays an essential role in B- and T- cell development (Bain and Murre, 1998; D'Cruz et al., 2010). Expression of miR-217 and miR-200a were found to be correlated with that of *TCF12* (Tables 5 and 6). Likewise, miR-194 was predicted to target TAB3 and expression level of TAB3 correlate with expression of miR-194 (Fig. 2A and Fig. 3A, Tables 5 and 6). Although there is no report on the interactions between these miRNAs and *TCF12*, *TAB3*, our result supports their interaction during NE response because, following NE induction, *TCF12* and *TAB3* expression in the intestine of non-infected line 6.3 control chickens was higher than that in line 7.2 control. A functional relationship between miR-499 and *TCF12* could not be observed in this study. In the spleen, the expression of both miR-34b and *TCF12* was decreased in line 7.2, supporting that miR-34b is not involved in *TCF12* regulation. *TCF12* may be targeted by miR-217 and miR-200a, whereas *BCL2* is targeted by both miR-200b and miR-429, as discussed above. These findings suggest multiple-and-one relationship between expression of miRNAs and their target genes, as previously described (Hashimoto et al., 2013).

The expression of key mRNA targets was also examined in the spleen. Although *BCL2*, *GJA1*, *TCF12*, *CXCR5* show a

Table 5

Summary of real-time PCR results for miRNAs in the intestine and spleen of line 6.3 and line 7.2 chickens.

miRNA	Target gene	miRNA expression			
		Intestine		Spleen	
		Line 6.3	Line 7.2	Line 6.3	Line 7.2
miR-215	CXCR5	—	+		
miR-429	BCL2	—	+		
miR-200b	BCL2	—	+		
miR-217	TCF12	—	+		
miR-200a	TCF12	—	+		
miR-34b	TCF12			+	—
miR-499	TCF12	+	—		
miR-1782	GJA1	+	—		
miR-194	TAB3	—	+		
miR-200b	VEGFA	—	+		
miR-429	VEGFA	—	+		
miR-1684	MAPK6			+	—

The "+" represents the up-regulated miRNAs and the "—" represents the down-regulated miRNAs in both lines. The blank indicates non-available RT-PCR data.

Table 6

Real-time PCR results for putative target genes of miRNA in the intestine and spleen of NE-infected and control chickens in both lines.

mRNA	Intestine				Spleen			
	NE-infected		Control		NE-infected		Control	
	Line 6.3	Line 7.2	Line 6.3	Line 7.2	Line 6.3	Line 7.2	Line 6.3	Line 7.2
CXCR5	+	—	0	0	+	—	0	0
BCL2	+	—	+	—	+	—	+	—
TCF12	+	—	+	—	+	—	0	0
GJA1	+	—	+	—	+	—	+	—
TAB3	+	—	+	—	0	0	0	0
VEGFA	0	0	+	—	0	0	0	0

The "+" represents the up-regulated miRNAs and the "—" represents the down-regulated miRNAs. The "0" indicates that mRNAs are equally expressed in both lines.

high expression in line 6.3 in comparison with line 7.2, evidence for their regulation seem to be absent once miRNA read count was out of harmony with mRNA expression (Fig. 3A, B, and Table 3). miRNAs predicted to target those genes are either stably expressed across two lines or not correlated with expression of target genes. Likewise, TAB3 and VEGFA that are equally expressed in both line 6.3 and line 7.2 were not regulated by miR-194 and miR-200b, miR-429 during NE infection. It is suspected that those genes possibly related to other miRNAs that are novel or were missed by the prediction tools.

In conclusion, our results show that a genetically determined disease phenotype to NE is associated with the expression of immune-related genes that are regulated by specific miRNAs. This new information lays the foundation for future studies to understand the functional relationship between these key NE-related mRNA targets and their corresponding miRNAs in NE.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2014.02.003>.

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